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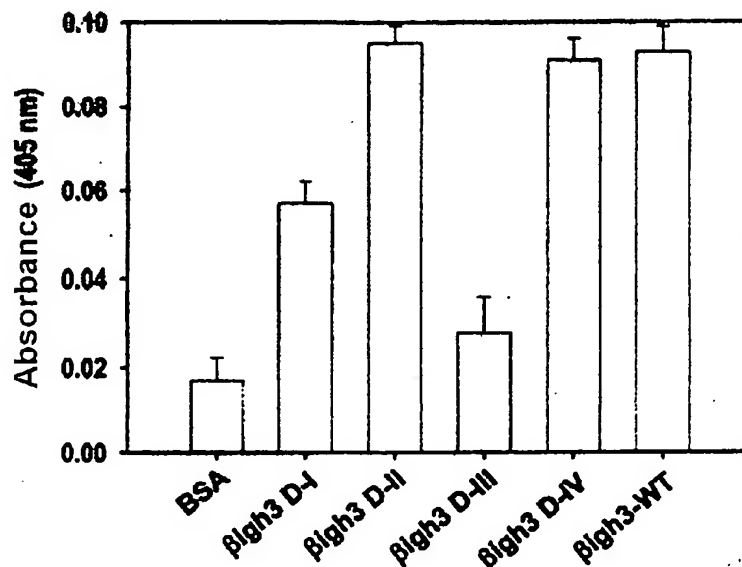
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(54) Title: METHOD FOR CELL ADHESION AND WOUND HEALING



(57) Abstract: The present invention relates to a method for cell adhesion and wound healing with internal domains of β ig-h3. Particularly, the present invention relates to the method of using recombinant proteins containing one or more of 2nd or 4th internal domain of β ig-h3 for cell adhesion and wound healing, wherein the 2nd or 4th internal domain of β ig-h3 has aspartic acid and isoleucine essential for interaction with integrin which represent a high homology in base sequence of β ig-h3 internal domains. The recombinant proteins containing one or more 2nd_i or 4th_i internal domain of β ig-h3 are effective for cell adhesion and wound healing by itself and can be used for developing cell culture medium and wound healing agent.

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METHOD FOR CELL ADHESION AND WOUND HEALINGFIELD OF THE INVENTION

5 The present invention relates to peptides for use in cell adhesion and wound healing. More particularly, the present invention relates to the use in cell adhesion and wound healing of peptides containing one or more copies of the 2nd and/or 4th fas-1 domain of β ig-h3, said 2nd and 4th
10 domains sharing a high homology in two amino acids, aspartic acid and isoleucine, essential for binding to integrin and thus mediating cell adhesion. Also, the present invention is concerned with an expression system for the peptides useful in cell adhesion and wound healing.

15

BACKGROUND OF THE INVENTION

β ig-h3 is an extracellular matrix protein whose expression is induced in various cell lines, including human
20 melanoma cells, mammary epithelial cells, keratinocytes, and lung fibroblasts, following signaling by active TGF- β (Skonier, J. et al., DNA Cell Biol. 13, 571, 1994). The β ig-h3 gene was first isolated by differential hybridization screening of a cDNA library made from a human lung
25 adenocarcinoma cell line that had been treated with TGF- β .

β ig-h3 gene encodes a 683-amino acid protein that is highly conserved between species. It contains an N-terminal secretory signal peptide and an Arg-Gly-Asp (RGD) motif at the C-terminus. The RGD motif is found in many
5 extracellular matrix proteins modulating cell adhesion and serves as a ligand recognition sequence for several integrins (Stonier, J. et al., DNA Cell Biol., 11, 511, 1992).

According to several studies, β ig-h3 is known to be
10 involved in cell growth and proliferation, wound healing, and cell adhesion, although the underlying mechanisms for these functions are still unclear. However, β ig-h3 seems to play an important role in the morphogenesis and interactions with cells and extracellular matrix proteins in various
15 tissues.

Some evidence related to the role of β ig-h3 in mediating cell attachment and detachment is provided by several studies. For example, purified β ig-h3 protein is found to promote the attachment and spreading of skin
20 fibroblasts while inhibiting the adhesion of A549, HeLa and Wi-38 cells in serum-free media. Particularly, β ig-h3 is known to have inhibitory activity against tumor cell growth, and to affect colony formation and morphology. The inhibitory activity was demonstrated by the report in which
25 transfection of β ig-h3 expression plasmids into CHO (Chinese

hamster ovary) cells led to marked decreases in cell proliferation and the ability of these cells to form tumors in nude mice. Further, a wound healing method was developed on the basis of the finding that application of a
5 pharmaceutically effective amount of β ig-h3 to wounds makes cells, especially fibroblasts, spread over and adhere to the wound site. Consequently, β ig-h3, a cell adhesion molecule induced by TGF- β in various cell lines, plays a very important role in cell growth, cell differentiation, wound
10 healing, morphogenesis and cell adhesion (Rawe, I. M. et al., Invest. Ophthalmol. Vis. Sci. 38, 893, 1997; Lebaron, R. G. et al., J. Invest. Dermatol. 104, 844, 1995).

β ig-h3 contains four 140 amino acid repeats with internal homology, namely fas-1 domains. The internal
15 repeat domains have highly conserved sequences found in secretory proteins or membrane proteins of various species, including mammals, insects, sea urchins, plants, yeasts, and bacteria. Proteins containing the conserved sequence are exemplified by periostin, fasciclin I, sea urchin HLC-2,
20 algal-CAM and mycobacterium MPB70. The conserved domain in these proteins (hereinafter referred to as "fas-1") consists of about 110 to 140 amino acids with two highly conserved branches, H1 and H2, of about 10 amino acids each (Kawamoto, T. et al., Biochem. Biophys. Acta. 1395, 288, 1998).

25 Four fas-1 domains are found in β ig-h3, periostin, and

fasciclin I, two fas-1 domains in HLC-2, and only one fas-1 domain in MPB70. Although the functions of the proteins are not elucidated clearly, some of them are known to act as cell adhesion molecules. For instance, β ig-h3, periostin, and fasciclin 1 are reported to mediate the adhesion of fibroblasts, osteoblasts, and nerve cells, respectively. Also, it is disclosed that algal-CAM is a cell adhesion molecule present in embryos of the algae Volvox (LeBaron, R. G., et al., J. Invest. Dermatol. 104, 844, 1995; Horiuchi, K. et al., J. Bone Miner. Res. 14, 1239, 1999; Huber, O. et al., EMBO J. 13, 4212, 1994).

At first, it was believed that the cell attachment activity of β ig-h3 would be mediated by the C-terminal RGD motif. However, some research results revealed that the RGD motif is not necessary for promoting the spreading of chondrocytes and that the mature soluble β ig-h3 whose RGD motif is deleted by carboxyl-terminus processing is able to inhibit cell adhesion, leading to the conclusion that the RGD motif of β ig-h3 is dispensable for mediating the cell attachment activity of β ig-h3. In addition, it has been recently reported that β ig-h3 promotes the spreading of fibroblasts via integrin α 1 β 1 whereas the RGD motif of β ig-h3 is not necessary for mediating the cell adhesion property of β ig-h3. According to a recent report, β ig-h3 binds specifically to integrin to enhance the cell adhesion and

spreading of cells irrespective of RGD motif (Ohno, S. et al., Biochim. Biophys. Acta 1451, 196, 1999). Further, the conserved peptides H1 and H2 of β ig-h3 were found to have no influence on β ig-h3-mediated cell adhesion. These results, taken together, indicate that amino acids indispensable for the cell attachment activity of β ig-h3 exist somewhere other than the H1 and H2 regions. A computer search based on homologies not only among the repeated fas-1 domains of β ig-h3 but also among fas-1 domains of other proteins revealed that there are a few highly conserved amino acids in addition to H1 and H2 peptides, suggesting the possibility of the involvement of the conserved amino acid sequences in the cell attachment activity.

Of the domains of β ig-h3, known to play an important role in cell adhesion, either of the 2nd or 4th domain is identified as a minimum domain essential for the cell adhesion of the molecule in accordance with the present invention. Based on these findings, recombinant proteins containing the essential functional domains are also identified as being effective for wound healing, in accordance with the present invention.

Recent research for wound healing has been subdivided into cell-biology and molecular biology and the promotion of wound healing has had increasing applications in various clinical fields. However, cell biological and molecular

biological mechanisms of wound healing still remain unclear. According to findings disclosed thus far, wound healing is a tissue response to trauma, leading to tissue repair through complex biological processes, including chemotaxis, cell
5 differentiation and replication, matrix protein synthesis, angiogenesis, and wound reconstitution (Steed, D. L., et al., Clin. Plast. Surg. 25, 397, 1998).

Growth factors are representative materials that appear in the early stage of the wound healing process and
10 control the subsequent wound healing process. Having strong influence over all stages of wound healing, growth factors act to control the growth, differentiation and metabolism of cells and reorganize the environs of the wound by their chemotactic properties which attract various cells types
15 that are involved in inflammation and tissue repair, cellular proliferation, stimulating angiogenesis and the synthesis and degradation of the extracellular matrix. PDGF (platelet-derived growth factor) attracts fibroblasts to the wound and stimulates them to proliferate, and transforming
20 growth factor-beta (TGF- β) causes them to make collagen. PDGF is chemotactic for most cells involved in wound healing, stimulates angiogenesis, remodeling and contraction, and activates wound healing cells (Mustoe, T. A. et al., J. Clin. Invest. 87, 694, 1991; Lepisto, J. et al., J. Surg. Res. 53,
25 596, 1992). EGF (epidermal growth factor) stimulates

keratinocyte migration, angiogenesis and granulation tissue development and activates mitogenesis of keratinocytes and fibroblasts (Franklin, J. D. et al., Plast. Reconstr. Surg. 64, 766, 1979; Buckley, A. et al., Proc. Natl. Acad. Sci. USA, 5 82, 7340, 1985). bFGF (basic fibroblast growth factor) stimulates angiogenesis, epithelialization, and collagenous fiber deposition, and associates with heparin in various forms to perform relevant functions (Tsuboi, R. et al., J. Exp. Med. 172, 245, 1990; Kinsnorth, A. N. et al., Br. J. 10 Surg. 77, 409, 1990). IGF (insulin-like growth factor) enhances cell differentiation. VEGF (vascular endothelial growth factor) increases vasopermeability and promotes endothelial mitogenesis.

Of the growth factors and cytokines involved in wound 15 healing, TGF- β is the most representative. Existing in three forms (TGF- β 1, TGF- β 2 and TGF- β 3) in mammals, the cytokine plays important roles in the growth and differentiation of various cells and has various complex functions, including control of cell growth, regulation of 20 immune responses, stimulation of osteogenesis, induction of cartilage specific macromolecules, and promotion of wound healing (Bennett, N. T. et al., Am. J. Surg. 165, 728, 1993). Appearing in the epithelium during wound healing, TGF- β is believed to stimulate the expression of integrin within 25 keratinocytes during re-epithelialization. In recent

research into TGF- β expression, it was revealed that TGF- β 3 mRNA is expressed in the epithelia of normal skin and acute and chronic wounds, while TGF- β 1 mRNA is not expressed in normal skin and chronic wounds, but expressed in the
5 epithelial layer regenerated from acute wounds, and nowhere is expressed TGF- β 2 mRNA (Schmid, P. et al., J. Pathol. 171, 191, 1993). Based on the effects, even though their mechanisms are not firmly established, TGF- β is expected to play a major role in re-epithelialization.

10 Expression of β ig-h3 is up-regulated by TGF- β , suggesting that β ig-h3 is involved in the mediation of some signals of TGF- β . CHO (Chinese hamster ovary) cells transformed with β ig-h3 expression plasmids are reported to show decreased tumorigenic ability (Skonier, J. et al., DNA
15 Cell Biol. 13, 571, 1994). In contrast, β ig-h3 expression is down-regulated in dexamethasone-treated stem cells, some tumor cells and the fibroblasts cultured from the skin lesion sites afflicted with localized hyperostosis of melorheostosis. β ig-h3 is also reported to serve as a
20 negative regulator of osteogenesis (Genini, M. et al., Int. J. Cancer 66, 571, 1996; Schenker, T. et al., Exp. Cell. Res. 239, 161, 1998; Kim, J. et al., J. Cell Biochem. 77, 169, 2000). In addition to these functions, β ig-h3, known as a cell adhesion molecule, promotes the adhesion and spreading
25 of fibroblasts in the dermis. According to studies into the

distribution of β ig-h3 in eye tissues, it is reported that the adhesion molecule is expressed in corneal epithelia of normal adults, intracorneal fetal stromal cells, and the endothelial and stromal cells in the process of wound healing. In addition, β ig-h3 is expressed in the juxtaglomerular apparatus and proximal tubules of the kidneys, and its expression is increased in diabetes mellitus. Further, it is found in subendothelial smooth muscles of the coronary arteries of normal persons, and its amount is increased in the endometria of blood vessels in the case of arteriosclerosis. However, the expression of β ig-h3 in normal dermal tissues and dermal wounds has not yet been firmly established (Klintworth, G. K. et al., Am. J. Pathol. 152, 743, 1998; Munier, F. L. et al., Nature Genetics 15, 247, 1997; Streeten B. W. et al., Arch. Ophthalmol. Vis. Sci. 38, 893, 1997). As mentioned above, the distribution and expression of β ig-h3 in normal human tissues remains unclear. Particularly, there are no reports regarding expression patterns of β ig-h3 in dermal wounds. However, some research groups have reported that β ig-h3 functions to promote the adhesion and spreading of dermal fibroblasts, so that it is expected to make a contribution to the promotion of wound healing.

SUMMARY OF THE INVENTION

With the background in mind, the intensive and thorough research on β ig-h3-mediated cell adhesion, leading to the present invention, resulted in the finding that there exist highly conserved amino acid sequences, in addition to H1 and H2 motifs, among fas-1 domains of β ig-h3 and among fas-1 domains of other peptides, as analyzed by computer search, and particularly, high homology is detected at aspartic acid and isoleucine residues at positions near the H2 region. In addition, the 2nd and 4th domains of β ig-h3, each containing the conserved amino acid residues, were found to induce cell adhesion through α 3 β 1 integrin. Further, recombinant proteins which were designed to have the 2nd and/or 4th fas-1 domain of β ig-h3 were identified as being identical to wild type β ig-h3 in cell attachment and spreading activity and wound healing effect.

Therefore, it is an object of the present invention to provide peptides which contain conserved amino acid sequences essential for cell attachment, spreading and detachment activity.

It is another object of the present invention to provide the use of the peptides in cell adhesion and wound healing.

It is a further object of the present invention to

provide an expression system for the peptides.

It is still a further object of the present invention to provide a method for attaching cells.

It is still another object of the present invention to
5 provide a method for healing wounds.

In accordance with an aspect of the present invention, there is provided a recombinant protein, comprising a portion of domains of β ig-h3, useful in mammalian cell attachment.

10 In accordance with another aspect of the present invention, there are provided expression vectors p β ig-h3 D-II, p β ig-h3 D-IV, and p β ig-h3 D-IV 4X, capable of expressing the 2nd and 4th fas-1 domain of β ig-h3 corresponding to amino acids 237-377 and 498-637,
15 respectively.

In accordance with a further aspect of the present invention, there are provided novel E. coli strains, transformed with the expression vectors p β ig-h3 D-II, p β ig-h3 D-IV, and p β ig-h3 D-IV 4X, identified as E. coli
20 BL21/His β -g (accession No. KCTC 0905BP), E. coli BL21/His β -e (accession No. KCTC 0904BP) and E. coli BL21/His β -e4x (accession No. KCTC 0906BP), respectively.

In accordance with still a further aspect of the present invention, there is provided a method for attaching
25 cells, comprising the steps of: preparing a recombinant

protein containing one or more copies of the 2nd and/or 4th domain of β ig-h3, by use of an expression vector; coating the recombinant protein onto a solid support; and applying cells to the protein-coated solid support.

5 In accordance with still another aspect of the present invention, there is provided the use of the recombinant protein in cell attachment.

 In accordance with yet another aspect of the present invention, there is provided the use of the recombinant
10 protein in wound healing.

 In accordance with still yet another aspect of the present invention, there is provided a method for healing wounds, comprising the steps of: coating a solid support with a recombinant protein containing one or more copies of
15 the 2nd and/or the 4th domain of β ig-h3; attaching skin cells to the solid support; and applying the solid support to wounds.

BRIEF DESCRIPTION OF THE DRAWINGS

20

Fig. 1a is a schematic diagram showing recombinant proteins β igh3-WT and β igh3- Δ RGD, wherein conserved regions are represented by \square and \boxtimes , and RGD motif by \circ .

Fig. 2 is a photograph showing SDS-PAGE results of
25 recombinant proteins β igh3-WT and β igh3- Δ RGD.

Fig. 3 is a microphotograph showing HCE cell adhesion and spreading effects of recombinant proteins β igh3-WT and β igh3- Δ RGD after dying with crystal violet.

Fig. 4 shows curves in which the HCE cell adhesion and spreading activities of the recombinant proteins β igh3-WT and β igh3- Δ RGD are found to be concentration-dependent as measured by the count (A) and surface area (B) of attached cells.

Fig. 5 shows histograms in which the HCE cell adhesion activities of the recombinant proteins β igh3-WT and β igh3- Δ RGD are compared in terms of count (A) and surface area (B) of attached cells.

Fig. 6a is a histogram showing effects of various compounds on the HCE cell adhesion activities of the recombinant proteins β igh3-WT and β igh3- Δ RGD.

Fig. 6b is a histogram showing effects of divalent cations on the HCE cell adhesion activities of the recombinant protein β igh3-WT.

Fig. 6c is a histogram showing the inhibition effect of anti-integrin monoclonal antibody on the HCE cell adhesion activity of the recombinant protein β igh3-WT.

Fig. 6d is a histogram showing the inhibitory effect of anti-integrin monoclonal antibody on the HCE cell adhesion activities of various proteins.

Fig. 6e is a histogram showing adhesion specificity of

K562 cells for the recombinant protein β igh3-WT and matrix proteins.

Fig 7 is a schematic diagram showing recombinant proteins having each of the fas-1 domains of β ig-h3.

5 Fig. 8 is a photograph showing SDS-PAGE results of recombinant proteins containing fas-1 domains of β ig-h3.

Fig. 9 is a histogram showing HCE cell adhesion activities of recombinant proteins containing fas-1 domains of β ig-h3.

10 Fig. 10 is a histogram showing the inhibitory effects of anti-integrin antibodies on HCE cell adhesion activities of the recombinant proteins containing fas-1 domains of β ig-h3.

Fig. 11 shows parts of amino acid sequences of various
15 matrix proteins containing fas-1 domains.

Fig. 12 is a schematic diagram showing substitution mutants of the 4th domain of β ig-h3.

Fig. 13 is a photograph showing SDS-PAGE results of recombinant substitution mutants of the 4th domain of β ig-h3.

20 Fig. 14 is a histogram showing cell adhesion activities of substitution mutants of the 4th domain of β ig-h3.

Fig. 15 is a schematic diagram showing recombinant proteins β igh3-D-IV, β igh3-D-IV 2X, 3X and 4X containing one,
25 two, three and four copies of the 4th domain of β ig-h3.

Fig. 16 shows photographs of the recombinant proteins β igh3-D-IV, β igh3-D-IV 2X, 3X and 4X run on 10% SDS-PAGE (A) and 8% nondenaturing PAGE (B), which are purified with the aid of Ni-NTA agarose resin

5 Fig. 17 shows optical photographs of wounds to which an ointment base is applied alone (A) and in combination with fibronectin (B), His- β -b (C), and β ig-h3-D-IV (D).

Fig. 18 shows microphotographs of wounds which are in the process of re-epithelialization after being treated with
10 an ointment base alone (A) and in combination with fibronectin (B), His- β -b (C), and β ig-h3-D-IV (D).

Fig. 19 shows optical photographs of wounds which have collagenous fibers formed after being treated with an ointment alone (A) and in combination with fibronectin (B),
15 His- β -b (C), and β ig-h3-D-IV (D).

Fig. 20 is a histogram showing HCE cell adhesion activities of the recombinant proteins β igh3-D-IV, β igh3-D-IV 2X, 3X and 4X, which contain at least one copy of the 4th domain of β ig-h3.

20 Fig. 21 shows optical photographs of wounds whose areas are reduced after being treated with a chitosan base alone (A) and in combination with fibronectin (B), β ig-h3 3X (C), and β ig-h3 4X (D).

DETAILED DESCRIPTION OF THE INVENTION

In the present invention, recombinant proteins are prepared on the basis of the 2nd and 4th fas-1 domains of β ig-h3 and used alone or in combination, for cell adhesion and spreading. To select the 2nd and 4th domains, the domains of β ig-h3 active in cell adhesion and spreading was identified. To this end, the C-terminal sequence Arg-Gly-Asp (RGD), known as a ligand recognition sequence for several integrins, was examined for its effect on the cell adhesion property of β ig-h3. The cell attachment activity was measured using the number and surface area of attached cells. As a result, β ig-h3 was found to promote cell adhesion and spreading, independent of the RGD motif.

Based on this finding, chemical reagents were used to address the specificity of cell adhesion activity of β ig-h3 and to get further clues about the nature of the cell surface receptor for β ig-h3. The data obtained from the use of chemical reagents suggest that the cell surface receptor for β ig-h3, which is involved in the cell adhesion activity of β ig-h3, could be one of the RGD-dependent integrins, which require divalent cations for interaction with β ig-h3.

Next, to identify minimum domains essential for the cell adhesion function of β ig-h3, an examination was made of the ability of each fas-1 domain to mediate cell adhesion.

This examination was based on the fact that fas-1 domains are found in various cell adhesion molecules, such as β ig-h3, periostin, fasciclin I, HLC-2, and algal-CAM and the number of fas-1 domains present in such adhesion molecules varies from protein to protein. This fact led to the inference that all four fas-1 domains might not be required for the cell adhesion activity of β ig-h3 and in an extreme case, only one domain could mediate the cell adhesion activity of β ig-h3. In the present invention, the either of 2nd or 4th fas-1 domain of β ig-h3 is revealed to be sufficient for the cell adhesion function of β ig-h3. These results lead to the conclusion that the H1 and H2 sequences, common in the four domains of β ig-h3, are not essential for the mediation of the cell adhesion activity of β ig-h3. Additionally, two amino acids, that is, aspartic acid and isoleucine at positions near the H2 region within the 2nd and 4th fas-1 domains, were found to be highly conserved, implying that these amino acid residues constitute a cell adhesion-related motif. The indispensability of the two conserved amino acids for cell adhesion was identified using substitution mutants of the 4th fas-1 domain of β ig-h3.

In another embodiment of the present invention, a wound healing method is provided in which the 2nd and 4th fas-1 domains of β ig-h3 are used, individually or in combination.

An comparison was made of wound healing effects of

mutant β ig-h3 proteins containing cell adhesion-active domains only and those of wild type β ig-h3 (β ig-h3-WT) containing domains a portion of the domains. In this regard, recombinant proteins containing the cell adhesion-active domains were applied to rats.

When a recombinant protein containing the 4th fas-1 domain of β ig-h3 was used a pharmaceutically effective ingredient for an ointment, wound shrinkage was observed, in addition to re-epithelialization and collagenous fiber formation. Ultimately, these results mean that one of the 2nd or 4th fas-1 domain of β ig-h3, in which the conserved aspartic acid and isoleucine exist, is useful for wound healing and thus can be utilized for the development of therapeutics for wounds.

Also, excellent cell adhesion and wound healing effects were obtained using a recombinant protein containing the 2nd fas-1 domain of β ig-h3 or 2nd and 4th domains.

Over the protein containing all of the domains, recombinant proteins containing parts of the domains have an advantage in that they can be produced in larger quantities because they are synthesized in water-soluble forms and thus do not undergo denaturation.

EXAMPLES

A better understanding of the present invention may be obtained in light of the following examples which are set forth to illustrate, but are not to be construed to limit the present invention.

5

EXAMPLE 1: Identification of Cell Adhesion Activity of RGD-Independent β ig-h3 Proteins

1-1: Production of recombinant β ig-h3 protein

10 In order to find the domains of β ig-h3 which have, in practice, cell adhesion and spreading activity, the C-terminal sequence Arg-Gly-Asp (RGD), known as a ligand recognition sequence for several integrins, was examined for effect on the cell adhesion property of β ig-h3. In this
15 regard, an RGD-deleted recombinant β ig-h3 protein (β igh3- Δ RGD) and a wild-type recombinant β ig-h3 protein (β igh3-WT) were prepared.

First, the full-length human β ig-h3 cDNA cloned in pBluescript (pBs β ig-h3) was digested with NdeI and BglII.
20 The DNA fragment was subcloned into the EcoRV-EcoRI site of pET-29b(+) (Novagen Inc.). β igh3-WT was prepared by introducing a 1351 bp NcoI fragment excised from β ig-h3 cDNA into the NcoI site of this clone. β igh3- Δ RGD was derived from β igh3-WT by cutting out a 3'-fragment of the β igh3-WT
25 plasmid with AocI and NotI followed by blunting and self

ligation, as shown in Fig. 1.

After being transformed with each recombinant plasmid, E.coli BL 21 DE3 was cultured in LB medium containing 50 µg/ml kanamycin at 37 °C until the optical density (OD) at 595 nm reached 0.5-0.6. The recombinant β ig-h3 proteins were induced using 1 mM isopropyl- β -D-(-)-thiogalactopyranoside (IPTG) at 37 °C for 3 hours. The pellet thus obtained was resuspended in a lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 1 mM PMSF, 0.5 mM DTT) and then sonicated. The inclusion bodies were dissolved in a denaturation buffer of 8 M urea containing 20 mM, followed by the purification of the denatured proteins with the aid of Ni-NTA resin (Qiagen). The recombinant proteins were eluted with 200 mM imidazole solution and then dialyzed sequentially from high to low urea in 20 mM Tris-HCl buffer containing 50 mM NaCl. These recombinant proteins were analyzed using SDS-PAGE, as shown in Fig. 2.

1-2: Assay for the cell adhesion activity of recombinant β ig-h3 fas-1 domain proteins

Human corneal epithelial (HCE) cells used in this assay were cultured in DMEM (EMEM/F-12, Gibco BRL) supplemented with 15% fetal bovine serum, Gentamicin (40

µg/ml), insulin (5 µg/ml), cholera toxin (0.1 µg/ml) and human epidermal growth factor (hEGF) at 37 °C in 5 % CO₂.

The cell adhesion assay was performed as follows. First, the recombinant β ig-h3 proteins or other
5 extracellular matrix proteins were let to adhere to the bottoms of 96-well microculture plates (Falcon) by incubation at 37 °C for 1 hour and blocked with PBS containing 0.2% BSA. The coated extracellular matrix proteins were human plasma vitronectin (Promega), purified
10 human plasma fibronectin (pFN), chicken collagen types I and II (Chemicon International Inc.), bovine collagen types IV and VI (Chemicon), mouse laminin (Chemicon), and bovine serum albumin (BSA) (Sigma). Cells were trypsinized and suspended in the culture media at a density of 2×10^5
15 cells/ml. 0.1 ml of the cell suspension was added to each well of the plates coated with the recombinant proteins.

Following incubation at 37 °C for 1 hour, unattached cells were removed by rinsing with PBS. Attached cells were incubated for 1 hour at 37 °C in 50 mM citrate buffer, pH
20 5.0, containing 3.75 mM p-nitrophenol-N-acetyl 1- β -D-glycosaminide as a hexosaminidase substrate and 0.25 % Triton X-100, followed by the addition of 50 mM glycine buffer, pH 10.4, containing 5 mM EDTA to block the enzyme activity. A measurement was made of absorbance at 405 nm in
25 a Multiskan MCC/340 microplate reader.

To determine cell area as an index for cell adhesion activity, 4×10^4 cells were applied to substrates in 48-well culture plates. The attached cells were fixed with 8 % glutaraldehyde (Sigma) and then stained with 0.25 % Crystal Violet (Sigma) in 20 % methanol. Measurement of cell areas was performed by Image-Pro plus software (Media Cybernetics). Experiments were repeated in triplicate with 200 or 300 measurements per site for each experiment. Data is reported as the mean area at specific time points \pm standard error of mean.

As a result of the measurement of cell adhesion and spreading activity using β ig-h3-WT and β ig-h3-ARGD, the numbers and surface areas of HCE cells which adhered to β ig-h3WT were clearly greater than those attached to albumin serving as a negative control, and were comparable to those of cells which adhered to fibronectin as shown in Fig. 3. The cell adhesion and spreading activities of β ig-h3 were concentration-dependent, as shown in Figs. 4A and 4B). β ig-h3-ARGD lacking the RGD motif was almost equally effective at supporting cell adhesion and spreading (Fig. 5A and 5B). These results, taken together, confirm that β ig-h3 supports cell adhesion and spreading, independent of the RGD motif.

EXPERIMENTAL EXAMPLE 1: Identification of Cell Surface Receptor of β ig-h3 Involved in Cell Adhesion Activity of

β ig-h3

1-1: Identification of cell adhesion activity using matrix peptide and reagent

In order to identify cell surface receptors involved
5 in the cell adhesion activity of β ig-h3 protein, an inhibition assay was performed using various reagents.

Initially, plastic culture dishes were coated with 10 μ g/ml fibronectin, β igh3-WT or β igh3-ARGD. HCE cells were preincubated for 30 min in media containing 5 mM EDTA, 100
10 μ g/ml β igh3-WT, 100 μ g/ml β igh3-ARGD, 1 mM RGD, 1 mM RGE or 100 μ g/ml fibronectin, or none of them, and then assayed for cell adhesion as in Example 1.

Cell adhesion to β ig-h3 was significantly inhibited by β ig-h3 itself, RGD peptide and EDTA, and partially inhibited
15 by fibronectin and EGTA, while being not inhibited by RGE peptide. Cell adhesion to fibronectin was also significantly inhibited by fibronectin itself, RGD peptide and EDTA, and partially inhibited by β ig-h3 and EGTA, but not by RGE peptide, as shown in Fig. 6A. These results
20 indicate that the cell surface receptor for β ig-h3, which is involved in the cell adhesion activity of β ig-h3, could be one of the RGD-dependent integrins.

1-2: Effect of divalent cations on cell adhesion activity

25 To analyze the divalent cation sensitivity of β ig-h3-

mediated adhesion, cells were suspended in HEPES-buffered saline (HBS) (150 mM NaCl, 25 mM HEPES, 2 mM EDTA, pH 7.4) at a density of 2×10^5 cells/ml and incubated at 37 °C for 30 min. Then, they were washed twice in HBS and resuspended in
5 the same buffer. Aliquots of cells (50 μ l) were then added to the microculture plate wells and incubated for 30 min at 37 °C in a humidified atmosphere of 5 % CO₂ with 50 μ l aliquots of HBS containing divalent cations (MnCl₂, MgCl₂ or CaCl₂) at a concentration twice as large as the final
10 concentration. Subsequently, they were plated on ligand-coated dishes to perform the adhesion assay.

Cell adhesion to β ig-h3 was strongly promoted by Mn²⁺, and to a lesser extent by Mg²⁺, but only marginally by Ca²⁺, as shown in Fig. 6B. taken together, the results
15 demonstrate that the cell surface receptor of β ig-h3 is a kind of RGD-dependent integrin which requires divalent cations for interaction with β ig-h3.

1-3: Identification of cell surface receptor of β ig-h3 using 20 monoclonal antibody against integrin

To identify receptors for β ig-h3, function-blocking monoclonal antibodies to integrin subunits were examined for their effect on the adhesion of HCE cells to a surface coated with β ig-h3. In this regard, initially, HCE (3×10^5
25 cells/ml) were preincubated in an incubation solution in

the presence of each of the monoclonal antibodies (5 $\mu\text{g/ml}$) against different types of integrins at 37 °C for 30 min. The preincubated cells were transferred onto plates precoated with $\beta\text{ig-h3}$ proteins and then incubated further at 5 37 °C for 1 hour, followed by the quantitative analysis of $\beta\text{ig-h3}$ binding with hexosaminidase substrate. The values are expressed as percentages of the number of cells adhering to $\beta\text{ig-h3}$ in the absence of monoclonal antibodies.

Adhesion to the $\beta\text{ig-h3}$ coated surface was specifically 10 inhibited by antibody against $\alpha 3$ subunit. Because the integrin $\alpha 3$ subunit is known to couple with the integrin $\beta 1$ subunit, anti- $\beta 1$ antibody significantly blocked cell adhesion to $\beta\text{ig-h3}$, as shown in Fig. 6C. Similar results were observed using HT1080 cells.

15 As a control experiment for the function-blocking antibodies, fibronectin, vitronectin, laminin and type I collagen were employed as substrata. HCE cells were preincubated with function-blocking monoclonal antibodies to integrin subunits and then transferred onto wells coated 20 with 10 $\mu\text{g/ml}$ fibronectin, vitronectin, type I collagen or laminin. Following incubation, cell counts of adhered cells were analyzed.

Cell adhesion to fibronectin was shown to be clearly inhibited by antibodies to integrins $\alpha 3$ and $\alpha 5$. Adhesion to 25 vitronectin and type I collagen was blocked by antibodies to

integrin αv and $\alpha 2$, respectively, whereas cell adhesion to laminin was inhibited by antibodies to integrins $\alpha 3$ and $\alpha 6$, as shown in Fig. 6D. On the other hand, antibody to $\beta 1$ integrin efficiently inhibited cells from adhering to all
5 ligands mentioned above.

For another control experiment, K562 cells, known to express $\alpha 5$, but not $\alpha 3$ integrin, were used. K562 cells were inoculated onto plates coated with β ig-h3-WT, fibronectin, laminin, or type I collagen and incubated for 1 hour,
10 followed by the hexosaminidase analysis. K562 cells did not adhere to β ig-h3, but adhered to fibronectin and vitronectin. Taken together, these results suggest integrin $\alpha 3\beta 1$ is a specific receptor for β ig-h3 in HCE cells, as shown in Fig. 6E.

15

EXAMPLE 2: Identification of Domains Essential to Cell Adhesion Activity of β ig-h3

In an attempt to identify essential amino acids
20 conferring cell adhesion activity of β ig-h3, an examination was made to determine whether each repeat domain is capable of mediating cell adhesion.

Four recombinant proteins corresponding respectively to four repeat domain were prepared: four β ig-h3 cDNA
25 fragments encoding amino acids 129-241, 237-377, 368-506,

and 498-637, respectively, were amplified by PCR and cloned into the EcoRV-XhoI site of pET-29b(+) and the resulting four expression vectors, named p β ig-h3 D-I, p β ig-h3 D-II, p β ig-h3 D-III, and p β ig-h3 D-IV, were used to prepare the
5 recombinant proteins, as shown in Fig. 7. E. coli transformants with the expression vectors p β ig-h3 D-II and p β ig-h3 D-IV were designated E. coli BL21/His β -g and E. coli BL21/His β -e and deposited in the Korean Collection for Type Culture of Korea Research Institute of Bioscience and
10 Biotechnology (KRIIBB) with accession Nos. KCTC 0905BP and KCTC 0904BP, respectively, on Dec. 4, 2000.

Expression and purification of the recombinant proteins β ig-h3 D-I, β ig-h3 D-II, β ig-h3 D-III, and β ig-h3 D-IV followed the procedure described in Example 1-1 and
15 they were identified by SDS-PAGE, as shown in Fig. 8.

In regard to the mediation of cell adhesion, the 2nd and 4th fas-1 domains were equally active compared to the wild type β ig-h3 whereas the 1st fas-1 domain was weak and the 3rd fas-1 domain was not active at all, as shown in Fig.
20 9.

In experiments with function-blocking antibodies to integrin subunits, both 2nd and 4th fas-1 domain-mediated cell adhesion were almost fully blocked by antibodies to α 3 and β 1 integrins, suggesting that both 2nd and 4th fas-1 domains
25 have amino acids essential for interacting with α 3 β 1

integrin, as shown in Fig. 10. These results also support the conclusion that neither H1 nor H2 sequence mediates cell adhesion activity of β ig-h3 because the 1st and 3rd domains are not active in cell adhesion, although they have H1 or H2 sequence.

EXAMPLE 3: Identification of Conserved Amino Acid Sequence Essential for Cell Adhesion of β ig-h3

3-1: Identification of Conserved Motif by Amino Acid Sequence alignment

To find the amino acid sequence responsible for cell adhesion in 2nd and 4th fas-1 domains of β ig-h3, which independently show cell attachment, a computer search based on homologies not only among the repeated fas-1 domains of β ig-h3, but also among fas-1 domains of other proteins was carried out. As a result, two amino acids, aspartic acid and isoleucine, near the H2 region, were found to be highly conserved among various proteins, as shown in Fig. 11. In addition, it was found that aspartic acid and isoleucine are both conserved in the 2nd and 4th fas-1 domains of β ig-h3, which are of high cell attachment activity, while only aspartic acid is conserved in the 1st fas-1 domain, which shows intermediate cell attachment activity. As for the 3rd fas-1 domain which shows no cell attachment activity, it has neither of the two amino acids. This fact is further

evidence that the aspartic acid and isoleucine residues near the H₂ region are indispensable for mediating the cell attachment and spreading activity.

5 3-2: Identification of cell adhesion activity of the
conversed amino acid sequence using substitution mutants

To further confirm that the two amino acids are essential for cell adhesion, the recombinant protein containing the 4th fas-1 domain of β ig-h3 was mutated by
10 substitution as shown in Fig. 12. The substitution mutant of β ig-h3 D-IV was prepared by PCR and its sequence was confirmed by base sequencing. The mutant protein was isolated and purified in the same manner as in Example 1-1 and confirmed on SDS-PAGE, as shown in Fig. 13.

15 Examination was made of the cell attachment activity of the mutated proteins wherein the Pro616, Asp617 and Ile618 of β igh3 D-IV were, in combination, substituted with Ser, Ala and Ser, respectively. The mutant protein having Ala instead of Asp617, named D617A (β igh3 D-IV-PaI) and the
20 mutant protein having Ser instead of Ile618, named I618S (β igh3 D-IV-PDs) significantly blocked cell adhesion whereas the mutant protein having Ser instead of Pro616, named P616S (β igh3 D-IV-sDI) was found to have no influence on cell adhesion activity. As for the mutant protein in which the
25 three amino acids were mutated, named P616S/D617A/I618S

(β igh3 DIV-sas), it also blocked cell adhesion, as shown in Fig. 14.

The nearly complete loss of the 1st fas-1 domain-mediated cell attachment activity in the 1st fas-1 domain mutated at Asp617 and Ile618 proved that the aspartic acid at position 617 and isoleucine at position 618 are very important in mediating the cell attachment activity of β igh3.

EXAMPLE 4: Identification of β igh3 Domains Effecting Wound Healing

4-1: Expression and purification of recombinant β igh3 protein

To examine whether only the β ig-h3 domains active in cell adhesion show the same wound healing function as that of the native β ig-h3 containing all four fas-1 domains, various recombinant β igh3 proteins were prepared as shown in Fig. 15: His- β -b containing all of 4 fas-1 domains; β igh3-D-IV containing the 4th domain alone; and β igh3-D-IV, 2X, 3X and 4X, each containing at least one 4th domain. Showing the same cell adhesion activity as in β igh3-WT prepared in Example 1, the recombinant β ig-h3 protein His- β -b was prepared from the recombinant expression vector pET-29 β anchoring at its EcoRV-EcoRI site an Asp718-BglIII fragment which was obtained by deleting a some amino-terminal region from β ig-h3 cDNA. The recombinant proteins His- β -b and

β igh3-D-IV were expressed and purified in the same manners as in Example 1-1 and 3.

The recombinant proteins containing at least one 4th domain, such as β ig3-D-IV, 2X, 3X and 4X were prepared as follows. A DNA fragment encoding to amino acid 498-637 corresponding the 4th domain was obtained by PCR and the PCR products were blunt-ended by Klenow enzyme. This blunt-ended cDNA fragment was inserted to the EcoRV site of the p β ig-h3 D-IV, which contained the 4th domain of β ig-h3, and the resulting expression vector was named p β I-h3 D-IV 2X. The insert of the p β ig-h3 D-IV 2X was excised by digestion with EcoRV and XhoI and blunt-ended by treatment with Klenow, followed by inserting the blunt-ended fragment into EcoRV sites of p β ig-h3 D-IV and p β ig-h3 D-IV 2X. The resulting expression vectors were named p β ig-h3 D-IV 3X and p β ig-h3 D-IV 4X. Expression of all recombinant proteins was induced for 3 hours in the presence of 1 mM IPTG and isolated by use of Ni-NTA resin (Qiagen). Isolated recombinant proteins were purified by elution with 20 mM Tris-HCl comprising 50 mM NaCl and 300 mM imidazole. β ig-h3 D-IV 2X, 3X and 4X can be produced in large amounts because they are synthesized as soluble forms, unlike β ig-h3 recombinant proteins containing all of the four domains, and do not undergo denaturation, as shown in Fig. 16A. Electrophoresis using non-denaturing gel revealed that β ig-h3 D-IV did not form polymers while 2X

partially formed polymers and 3X and 4X each readily formed polymers , as shown in Fig. 16B.

E. coli BL21/His β -e4X, which harbors the expression vector p β ig-h3 D-IV 4X containing four 4th domains of β ig-h3, 5 was deposited in the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) with accession No. KCTC 0906BP on Dec. 4, 2000.

Fibronectin, serving as a positive control, was purified from citrated rat plasma by affinity chromatography 10 using gelatin-sepharose 4B. The plasma was filtered at room temperature through non-substituted sepharose 4B and the eluate was loaded onto gelatin sepharose 4B equilibrated with 0.05 M Tris-Cl containing 0.05 M EACA (ϵ -amino caproic acid), 0.02 M sodium citrate and 0.02 % sodium azide. After 15 being eluted, most plasma proteins were washed with a buffer containing 1 M sodium chloride. Then, absorbed fibronectin was eluted with 3M uric acid isotonic buffer which was subsequently dialyzed for about 48 hours against PBS, pH 7.2, to purify fibronectin. Its concentration was determined by 20 UV absorbance at 280 nm and freeze-dried before being stored at -20 °C.

4-2: Assay for wound healing activity of β ig-h3 D-IV containing the 4th domain

25 To compare wound healing activity between the

recombinant β ig-h3 protein His- β -b, which contains all of the four fas-1 domains, like native β ig-h3 protein, and the recombinant β ig-h3 protein β igh3-D-IV, which contains the 4th domain alone, ointment bases comprising the recombinant
5 proteins were tested as follows.

Four dermal whole layer wounds, each 2 cm in diameter, were made on the backs of rats and divided into test groups 1-A, 1-B, 1-C and 1-D according to the ointment applied thereto.

10 1-A: coated at a dose of 1 gm per day with an ointment base combined with no materials..

1-B: coated at a dose of 1 gm per day with an ointment in which fibronectin was combined at a concentration of 100 μ g/ml with a base.

15 1-C: coated at a dose of 1 g per day with an ointment in which His β -b protein was combined at a concentration of 100 μ g/ml with a base.

1-D: coated at a dose of 1 g per day with an ointment in which β igh3-D-IV protein was combined at a concentration
20 of 100 μ g/ml with a base.

The backs of etherized rats were shaved, followed by sterilizing the shaved region with betadin solution. In test group 1, the back of each rat was cut by use of a No. 15 surgical blade to form four circular wounds with a
25 diameter of 2 cm penetrating the whole dermal layers.

Ointments for test groups 1-A, 1-B, 1-C and I-D were applied at an amount of about 1 g to the wounds which were then covered with a synthetic dressing (Tegaderm® 3M) and lightly bandaged. Application of ointments was performed once every
5 day.

With a base of an aqueous material (SamA base), each of the ointments contained, per 1 g, spermaceti 38 mg, stearyl alcohol 116 mg, polyethylglycol 38 mg, conc. glycerin 192 mg, ethanol 23 mg, lauryl sodium sulfate mg,
10 ethyl paraoxybenzoate 0.87 mg, butyl paraoxybenzoate 0.12 mg, and purified water.

First, morphologies of wounds were observed. The same scale was positioned near each wound and pictures were taken at the same distance from each wound. Pictures were scanned
15 in a computer and used to measure areas of the wounds with the aid of NIH image analysis system (Bio-Optics). To take the pictures, the muscle was completely relaxed by etherizing the rats. Measurements were performed once every other day until the 22nd day. For comparing test groups, the
20 measured values were analyzed according to ANOVA test and Scheffer's test.

In all test groups, wound areas were observed to be gradually reduced just after the formation of wounds. The test groups to which fibronectin and the recombinant β ig-h3
25 protein were applied were measured to be more quickly

reduced in wound area than the test group to which the ointment base alone was applied. A significant difference in wound area was seen after 7 days of ointment application. Statistically, there were significant differences ($p < 0.05$) between wounds of group I-A and the other groups, which did not show a significant difference therebetween. The results are given in Table 1, below and Fig. 17.

TABLE 1: Healing Effect of Ointment Bases Combined with Recombinant β ig-h3 Proteins on Wounds

Group	Day											
	0	2*	4	6	8*	10*	12	14	16	18	20	22
I-A (Control)	3.15 ± 0.49	3.09 ^a ± 0.31	2.45 ± 0.39	2.17 ± 0.46	1.64 ^a ± 0.50	1.80 ^a ± 0.11	0.84 ± 0.32	0.56 ± 0.31	0.34 ± 0.07	0.19 ± 0.04	0.17 ± 0.05	0.08 ± 0.01
I-B (Fibronectin)	3.17 ± 0.78	2.38 ^b ± 0.55	2.01 ± 0.54	1.83 ± 0.42	1.39 ^b ± 0.38	1.26 ^b ± 0.18	0.54 ± 0.11	0.39 ± 0.12	0.25 ± 0.12	0.16 ± 0.09	0.12 ± 0.09	0.06 ± 0.03
I-C (His- β -b)	3.14 ± 0.46	2.58 ^b ± 0.47	1.89 ± 0.26	1.71 ± 0.33	1.42 ^b ± 0.45	1.37 ^b ± 0.71	0.46 ± 0.06	0.36 ± 0.13	0.26 ± 0.09	0.15 ± 0.10	0.11 ± 0.03	0.03 ± 0.01
I-D (β ig-h3-D-IV)	3.15 ± 0.43	2.62 ^b ± 0.52	2.37 ± 0.45	1.98 ± 0.52	1.51 ^b ± 0.21	0.98 ^b ± 0.69	0.44 ± 0.24	0.22 ± 0.09	0.20 ± 0.09	0.18 ± 0.10	0.13 ± 0.07	0.06 ± 0.01

Value : mean \pm SD

*: $p < 0.05$ by ANOVA and Scheffe's test

a, b: vertically significant difference of data in statistics

15

Histological analysis was conducted under an optical microscope. Biopsies of wound sites were taken at days 3, 7, 10, 14 and 20, and fixed in 10 % formalin and solidified with paraffin. 6 μ m slices of the samples were dyed with hematoxylin-eosin (H&E) and Masson's trichrome before

observation under a microscope. Wound healing effects according to time of each test material were evaluated through re-epithelialization and formation of collagenous fibers. In the case of re-epithelialization, epithelial formation was semi-quantified in such a way that zero was set for the formation of no epithelial layers, 1+ for initiation of epithelialization, 2+ for incomplete epithelial layer structure, and 3+ for complete epithelial layer structure. Regarding comparison among test groups and differences according to time within each group, measured values were statistically analyzed using ANOVA test and Scheffe's test. As for collagenous fiber formation, it was graded as 1+ for insignificant formation of collagenous fibers as observed with trichrome dye, 2+ for scatteringly spaced collagenous fibers, and 3+ for dense collagenous fibers.

As observed by an optical microscope, re-epithelialization appeared to start at day 7 to 10 in test groups 1-B, 1-C and 1-D and be completed at day 20. In the case of the control group 1-A, on the other hand, re-epithelialization was not yet initiated even at day 14 and was not completed at day 20. The results are given in Table 2, below and Fig. 18.

TABLE 2: Re-epithelialization of Wound

Group	Day				
	3	7	10	14	20
1-A (Control)	0	0	0	0	2+
1-B (Fibronectin)	0	1+	1+	2+	3+
1-C (His- β -b)	0	0	1+	2+	3+
1-D (β igD-IV)	0	0	1+	2+	3+

Results for formation of collagenous fibers are given in Table 3, below. As seen in Table 3, collagenous fibers were not significantly formed in test groups 1-A and 1-D until day 7 with maintenance of grade +1, whereas test groups 1-B and 1-C were graded as 2+. However, all test groups were graded as 2+ at day 10 with relatively rich collagenous fibers. At day 14, it was observed that collagenous fibers were densely formed and well arranged with grade +3, as shown in Fig. 19. Naturally, denser collagenous fibers reflect more improved wound healing progress.

TABLE 3: Formation Behavior of Collagenous Fibers at Wound

Group	Day				
	3	7	10	14	20
1-A (Control)	1+	1+	2+	3+	3+
1-B (Fibronectin)	1+	2+	2+	3+	3+
1-C (His- β -b)	1+	2+	2+	3+	3+
1-D (β igD-IV)	1+	1+	2+	3+	3+

Formation grade of collagenous fibers

0 : negative, 1+ : insignificant,
 2+ : scatteringly formed, 3+ : very dense

4-3: Wound healing effect of at least one 4th domain-containing recombinant proteins β igh3-D-IV, β ig-h3 D-IV 2X, 3X and 4X

5 Based on the finding that β igh3-D-IV containing only the 4th domain is efficient for wound healing, β igh3-D-IV 2X, 3X and 4X, which contained the 4th domain in duplicate, triplicate and quadruplicate, respectively, were prepared in order to assay for wound healing activity.

10 The recombinant proteins were assayed for HCE cell adhesion activity in the same manner as in Example 1-2. The results are given in Fig. 20. As seen, the recombinant proteins β ig-h3 D-IV 2X, 3X and 4X were all found to effectively induce the adhesion of HCE cells.

15 In order to examine wound healing effects of the recombinant proteins, the following experiments were conducted.

Adult Sprague-Dawley lineage rats with a body weight of 250-300 gm were raised with standard feedstuff at a
20 constant temperature and humidity.

In test group 2, four circular dermal whole layer wounds were made on the back of each rat and coated with chitosan bases combined with materials of interest:

2-A: wound coated with chitosan base only

25 2-B: wound coated with chitosan base in combination

with 500 µg/ml of fibronectin

2-E: wound coated with chitosan base in combination
with 500 µg/ml of β ig-h3 D-IV 3X protein

2-F: wound coated with chitosan base in combination
5 with 500 µg/ml of β ig-h3 D-IV 4X protein

The composites based on chitosan were prepared as follows. 1 g of water soluble chitosan (poly(1-4) 2-amino-2-deoxy- β -D-glucan) with a molecular weight of 600,000 Da was dissolved in 100 ml of sterile distilled water and the
10 resulting 1% solution was dispensed in aliquot of 2 ml to to 12-well plate (Corning, USA), followed by the addition of 100 µg of gentamycin per well. Fibronectin, β igh3-D-IV. 3X, and β igh3-D-IV 4X were individually added to a concentration of 500 µg/ml and frozen at -70 °C, followed by freeze-drying
15 in a freeze drier (Ilshin) for 12 hours to give disc-shaped composites.

The backs of etherized rats were shaved, followed by sterilizing the shaved region with betadin solution. Penetrating the whole dermal layers, four circular wounds
20 with a diameter of 7 mm were formed on the back of each rat. The wounds were covered with composites used for test groups 2-A, 2-B, 2-E and 2-F, respectively, and then with Tegaderm® (3M) and lightly bandaged. The composites were changed with fresh ones every three days.

25 Wound healing effects were evaluated by determining

appearances of the wounds as in Example 4-2.

A high wound healing effect was obtained from the composite containing the recombinant protein β ig-h3 D-IV 3X or 4X.

5 All rats, except for all members in the test group 2-A, one in the test group 2-B and two in test groups 2-E and 2-F each, were completely recovered from the wound at day 12 to 15. All wound areas reduced in size just after the formation of wound. As for the test group 2-A, its wound
10 area was observed to be reduced at a relatively slow rate throughout the period of healing time. In the other test groups, the wound areas were reduced greatly in the first three days, gradually to day 9, and then greatly again. Turning to comparison among wounds, there were more
15 significant differences ($p < 0.05$) for the whole period of 15 days in the test groups 2-B, 2-E and 2-F, than in the test group 2-A, as shown in Table 4 and Fig. 21.

TABLE 4: Reduction of Wound Area (mm^2)

20

Group	Day					
	0	3*	6*	9*	12*	15*
2-A (Chitosan)	49.3 \pm 4.0	34.5 \pm 0.6 ^a	26.1 \pm 0.5 ^a	13.8 \pm 0.5 ^a	10.8 \pm 0.3 ^a	3.4 \pm 0.2 ^a
2-B (Chitosan+Fibronectin)	49.2 \pm 0.5	24.1 \pm 0.6 ^b	12.9 \pm 0.6 ^b	9.7 \pm 0.8 ^b	3.2 \pm 0.4 ^b	0.8 \pm 0.2 ^b
2-C (Chitosan+ β ig-h3 3X)	49.2 \pm 1.5	25.3 \pm 0.7 ^b	16.6 \pm 0.6 ^b	11.2 \pm 0.5 ^b	5.0 \pm 0.8 ^b	1.2 \pm 0.2 ^b
2-D (Chitosan+ β ig-h3 4X)	48.5 \pm 0.4	24.5 \pm 0.6 ^b	14.1 \pm 0.7 ^b	9.6 \pm 0.6 ^b	4.2 \pm 0.3 ^b	1.1 \pm 0.2 ^b

Value: Mean \pm S.D

*: $p < 0.05$ by ANOVA test

a, b : significant difference of data in statistics

5 Consequently, the recombinant proteins of the present invention, which contain the 2nd and 4th domains of β ig-h3, alone or in combination, or in multiplicate are effective for cell adhesion and wound healing and ultimately can be utilized in developing cell culture and wound healing agents.

10

INDUSTRIAL APPLICABILITY

 In the present invention, there are provided recombinant proteins containing at least one of the 2nd and
15 4th domains of β ig-h3 in which one aspartic acid and one isoleucine residue, known to be essential for association with integrin, are highly conserved. Also, the recombinant proteins themselves are useful for cell adhesion and wound healing, making a contribution to the development of cell
20 culture methods and wound healing agents.

 The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation. Many modifications
25 and variations of the present invention are possible in

light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

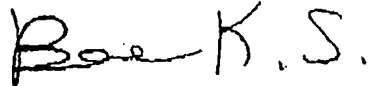
INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : KIM, In-San

Dongseotown 106-908, #1040, Maeho-dong, Soosung-ku, Daegu 706-140,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Escherichia coli</i> BL21/His β -e	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0904BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input checked="" type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on December 04 2000 .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):  BAE, Kyung Sook, Director Date: December 07 2000

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

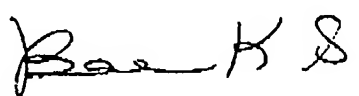
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RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : KIM, In-San

Dongseotown 106-908, #1040, Maehong-dong, Soosung-ku, Daejeon 305-140,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Escherichia coli</i> BI.21/His β -g	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: KCTC 0905BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input checked="" type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on December 04 2000 .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejeon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):  BAE, Kyung Sook, Director Date: December 07 2000

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

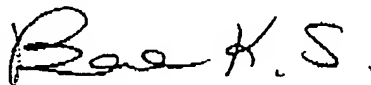
INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

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TO : KIM, In-San

Dongseontown 106-908, #1040, Maeho-dong, Soosung-ku, Daegu 706-140,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Escherichia coli</i> BL21/His β -e4X	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0906BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input checked="" type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
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Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):  BAE, Kyung Sook, Director Date: December 07 2000

What is Claimed is:

1. A method of for attaching mammalian cell with a recombinant protein comprising a portion of domains of β ig-h3.
5 h3.

2. The method according to claim 1, wherein the recombinant protein comprises the 2nd fas-1 domain of β ig-h3, the 4th fas-1 domain of β ig-h3, or both of the 2nd and 4th domains of β ig-h3.
10

3. The method according to claim 2, wherein the 2nd or 4th domain of β ig-h3 exists as a single copy or in multiple copies.
15

4. The method according to claim 2, wherein the 2nd fas-1 domain of β ig-h3 comprises the amino acid sequence 237-377 of β ig-h3 and is expressed from the expression vector p β ig-h3 D-II.
20

5. The method according to claim 2, wherein the 4th fas-1 domain of β ig-h3 comprises the amino acid sequence 498-637 of β ig-h3 and is expressed from the expression vector p β ig-h3 D-IV.
25

6. The method according to claim 3, wherein the recombinant protein comprises the amino acid sequence 502-637 of β ig-h3 in tetraplicate and is expressed from the expression vector p β ig-h3 D-IV 4X.

5

7. An expression vector p β ig-h3 D-II, comprising amino acids 237-377 of β ig-h3 and capable of expressing the 2nd fas-1 domain of β ig-h3.

10

8. A novel E. coli strain, transformed with the expression vector of claim 7, identified as E. coli BL21/His β -g (accession No. KCTC 0905BP).

15

9. An expression vector p β ig-h3 D-IV, comprising amino acids 498-637 of β ig-h3 and capable of expressing the 4th fas-1 domain of β ig-h3.

20

10. A novel E. coli strain, transformed with the expression vector of claim 9, identified as E. coli BL21/His β -e (accession No. KCTC 0904BP).

25

11. An expression vector p β ig-h3 D-IV 4X, expressing recombinant protein which comprises the amino acid sequence 502-637 of β ig-h3 in tetraplicate and multiple copies of the 2nd or the 4th fas-1 domain of β ig-h3.

12. A novel *E. coli* strain, transformed with the expression vector of claim 11, identified as *E. coli* BL21/His β -e4x (accession No. KCTC 0906BP).

5

13. The method according to claim 1, comprising the steps of:

1) preparing a recombinant protein comprising one or more copies of the 2nd and/or 4th domain of β ig-h3, by use of
10 an expression vector;

2) coating the recombinant protein onto a solid support; and

3) applying cells to the protein-coated solid support.

15 14. A method for healing mammalian wound with a recombinant protein comprising a portion of domains of β ig-h3.

20 15. The method according to claim 14, comprising the steps of:

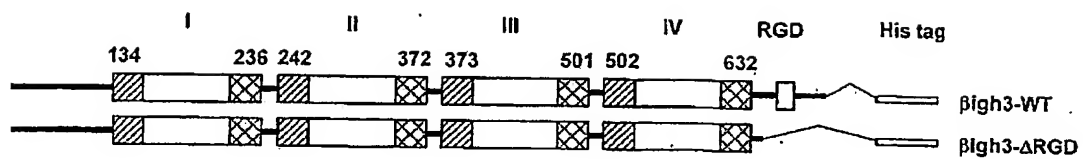
1) coating a solid support with a recombinant protein containing one or more copies of the 2nd and/or the 4th domain of β ig-h3;

2) attaching cells to the solid support; and

25 3) applying the solid support to wounds.

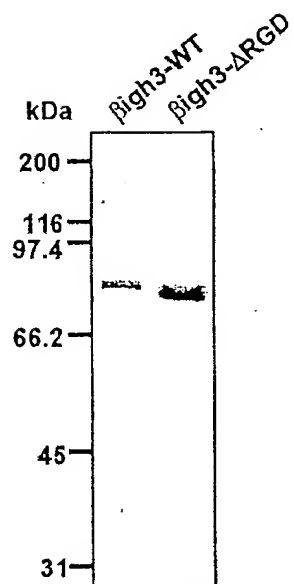
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FIG. 1



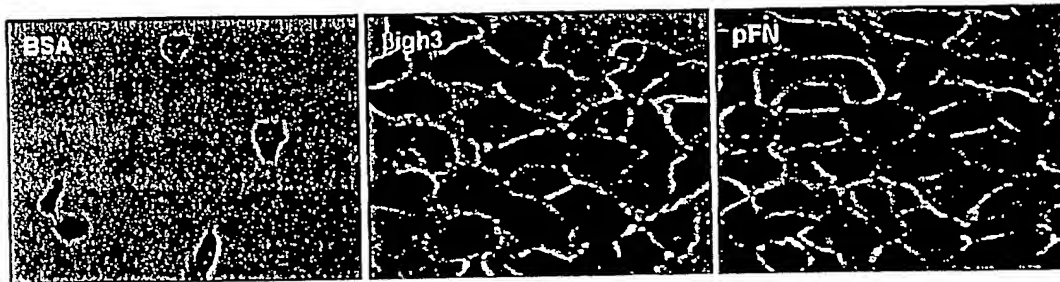
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FIG. 2



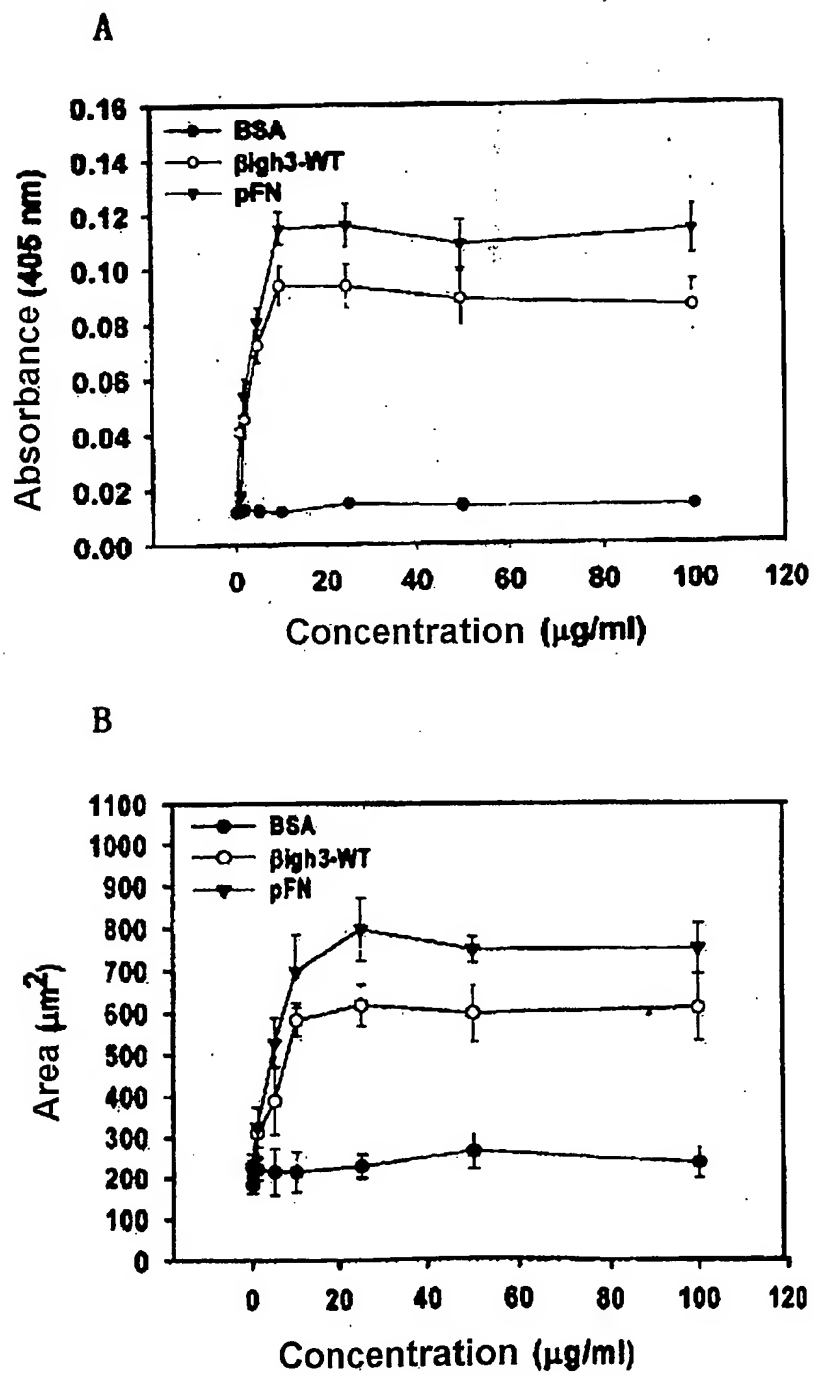
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FIG. 3



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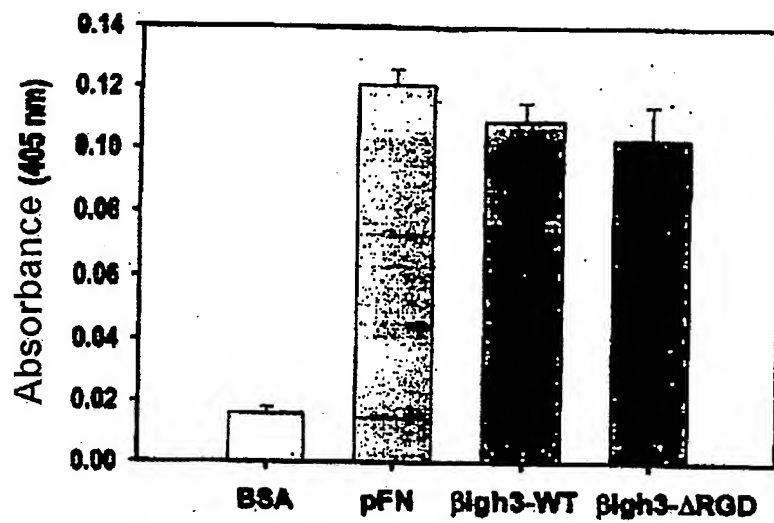
FIG. 4



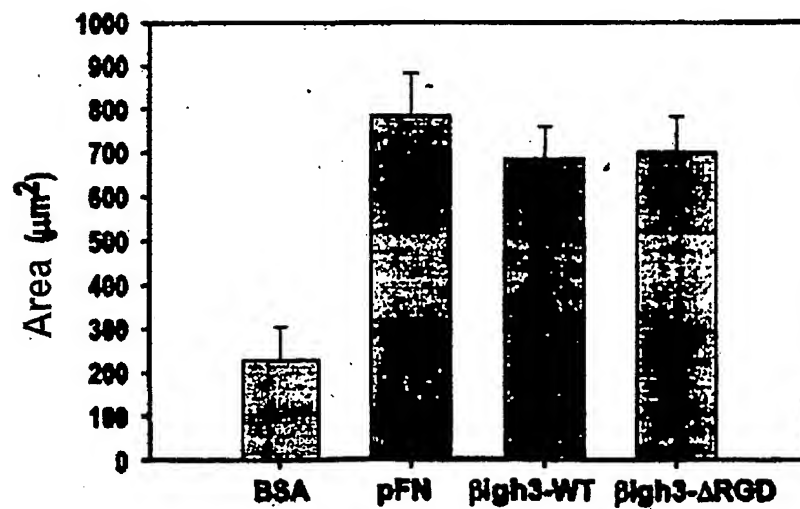
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FIG. 5

A



B



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FIG. 6a

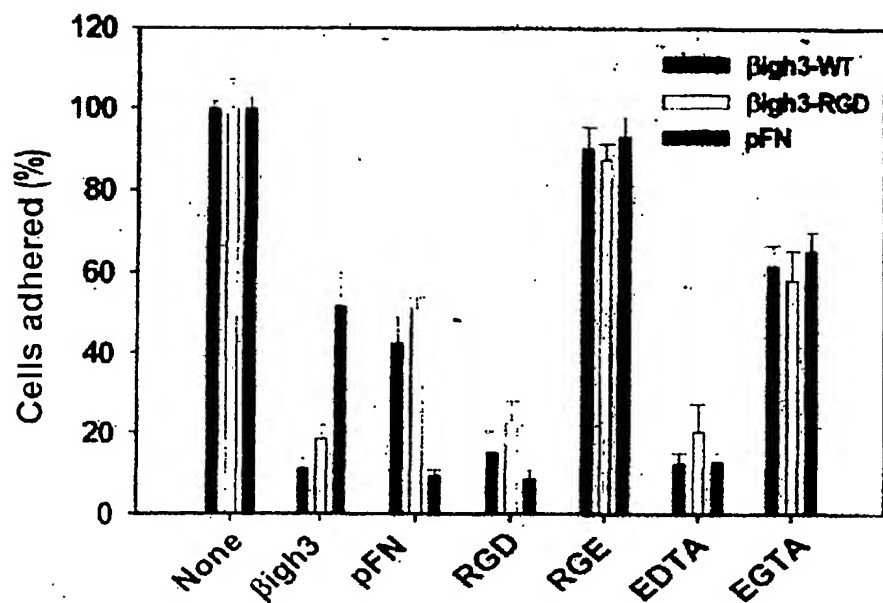
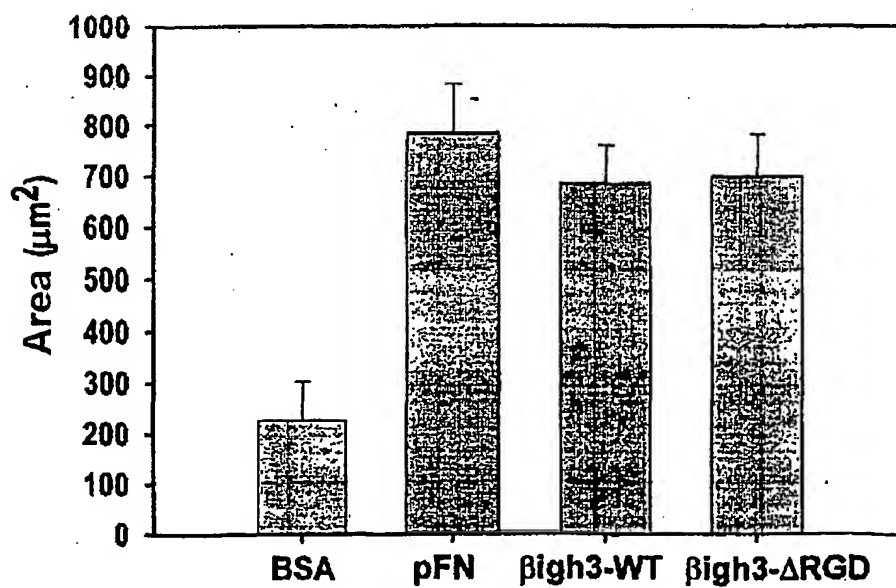
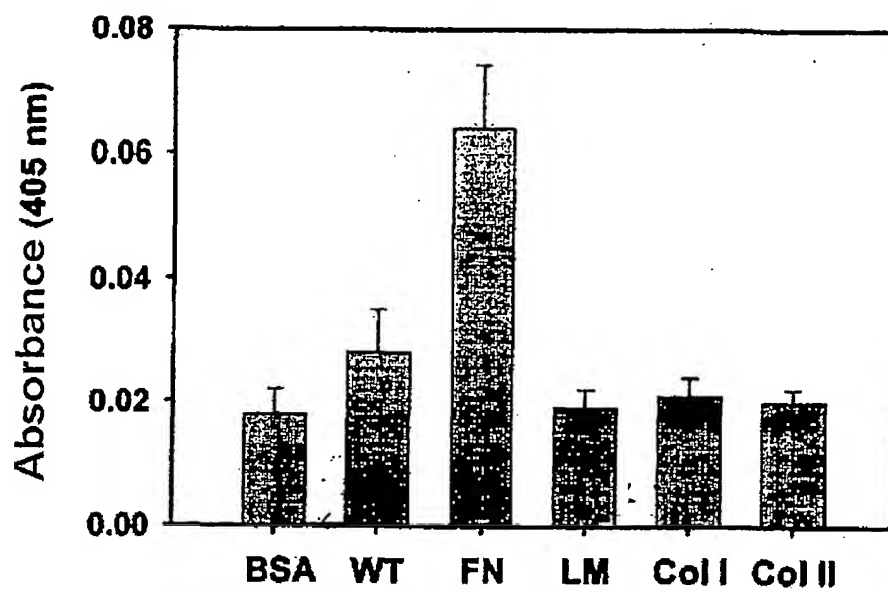


FIG. 6b



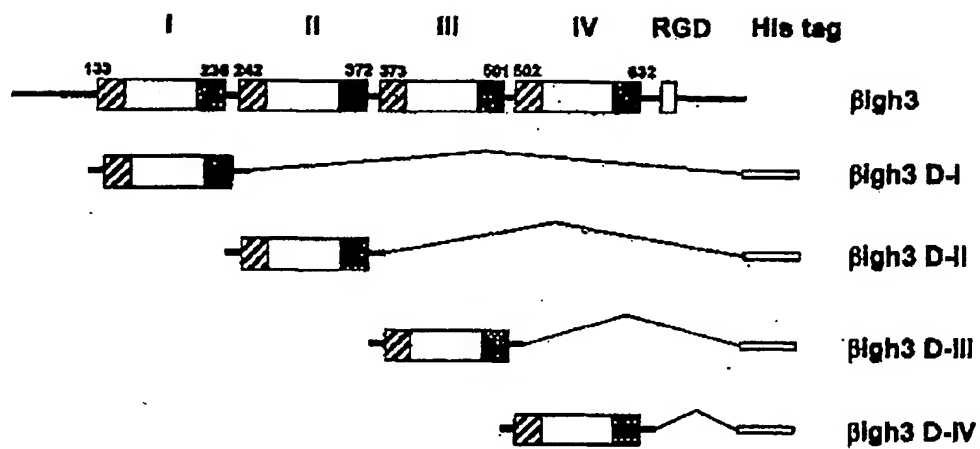
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FIG. 6e



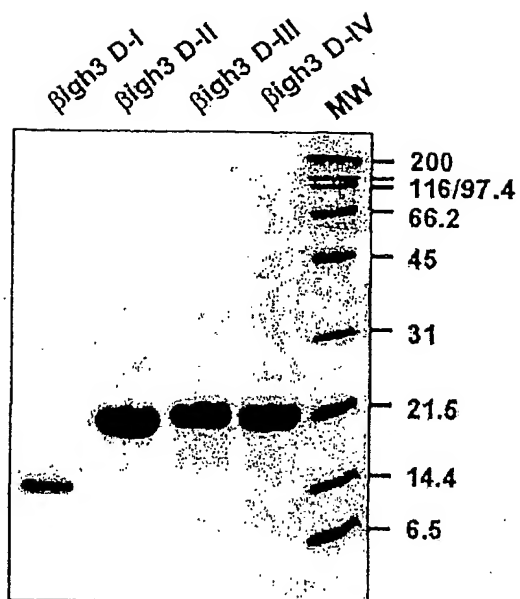
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FIG. 7



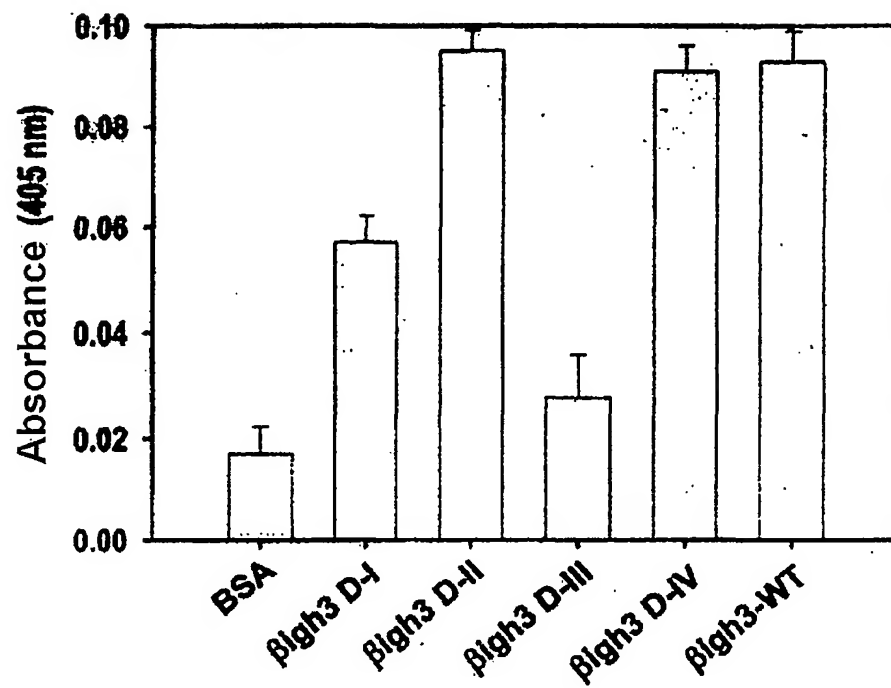
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FIG. 8



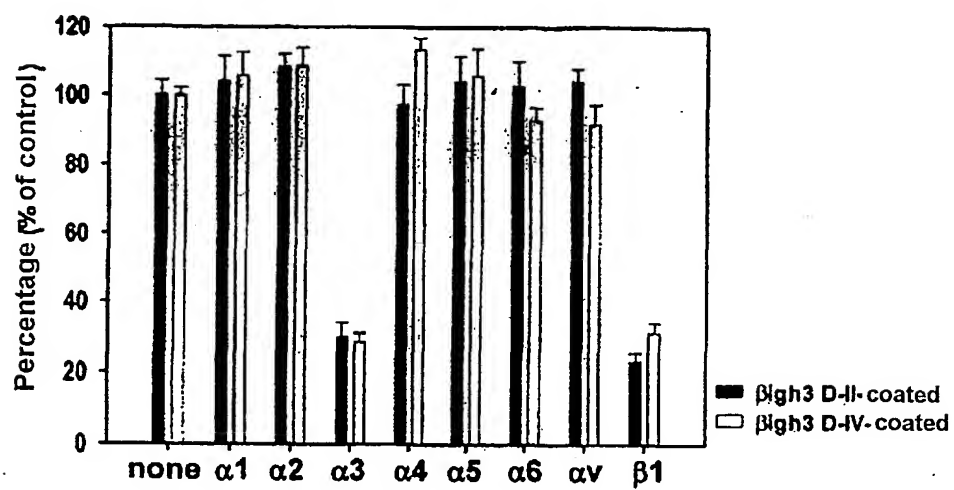
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FIG. 9



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FIG. 10



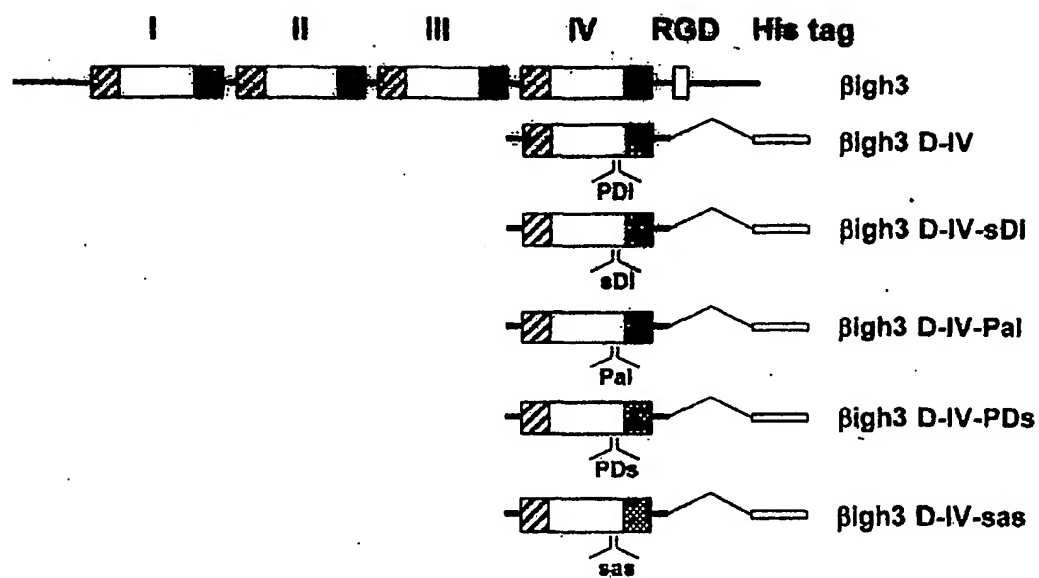
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FIG. 11

		B2	
BIGH3_HUMAN	211	TVNCARLLKADHHATNGVVHLIDKVI...	236
BIGH3_PIG	211	TVNCARLLKADHHATNGVVHLIDKVI...	236
BIGH3_CHICK	201	TVNCARLLKADHHATNGVVHVIDKVI...	228
BIGH3_HUMAN	346	INGKAIISNKDILATNGVIHYIDELLI..	372
BIGH3_PIG	346	INGKPIISNKDVLATNGVIHFIDELLI..	372
BIGH3_CHICK	338	LNGRAIIANKDILATNGVVHFVNELLI..	364
OSF2_HUMAN	340	VNGIKMVNKKDIVTNNGVIHLIDQVLI..	366
OSF2_MOUSE	342	INGIKMVNKKDIVTKNGVIHLIDEVLI..	368
BIGH3_HUMAN	608	VNKEPVAE-PDIMATNGVVHVITNVL...	632
BIGH3_PIG	608	VNKEPVAE-ADIMATNGVVHTINTVL...	632
BIGH3_CHICK	600	VNKEPVAE-SDIMATNGVIHAVSSVL...	624
SLL1735 homolog	106	VKNATVLA-ADIEADNGIIHVIDNVILMG	133
SLL1735	106	VKNATVIL-PDIEADNGIIHVIDNVILMG	133
SLL1483	152	VNKATVIS-ADV DASNGVIHVIDQVIL..	177
OSF2_HUMAN	604	VNELKSKE-SDIMTTNGVIHVVDKLL...	628
OSF2_MOUSE	606	VNELKSKE-SDIMTTNGVIHVVDKLL...	630
Midline Fasciclin	543	INNLAIIIDADIMGTNGVLHVIDTIL...	568
HLC-32	341	-SKASRVILRDIPTTNGVIQVIDRVIL..	366
Midline Fasciclin	825	KIENAGVTKCDVVATNGILHEINDIIV..	851
HLC-32	196	TANGARVVEADRKASSGLIHVVDKVI...	221
consensus		VNNAARVVKADZTHATNGVIHVIDKVLIMG	

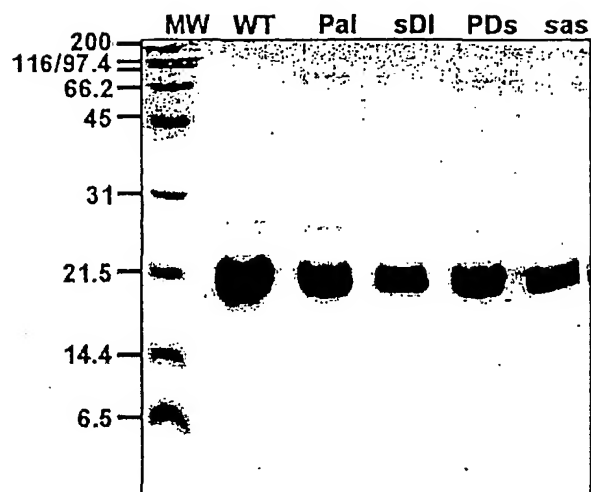
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FIG. 12



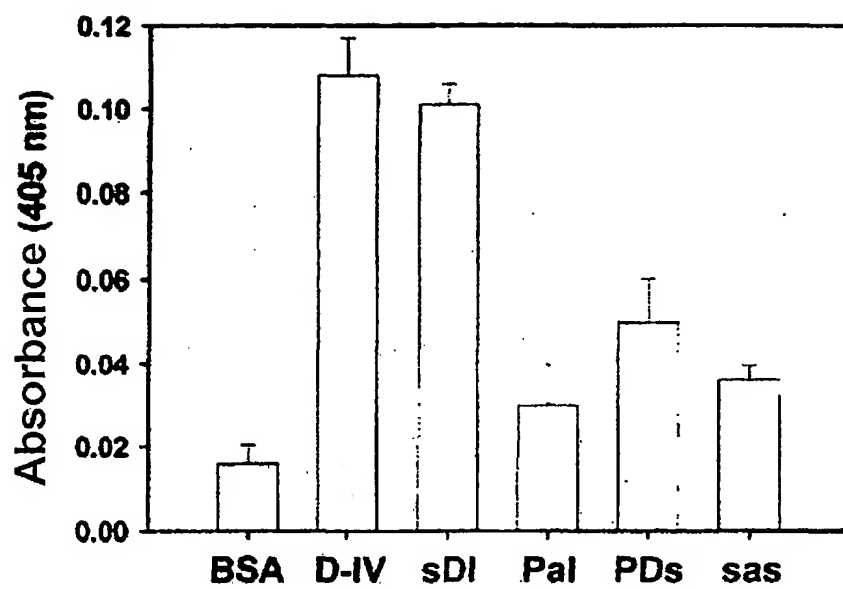
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FIG. 13



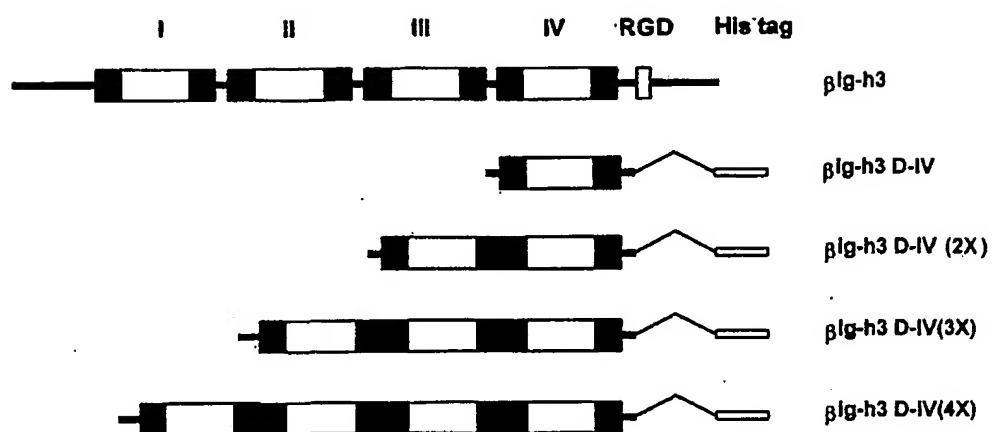
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FIG. 14



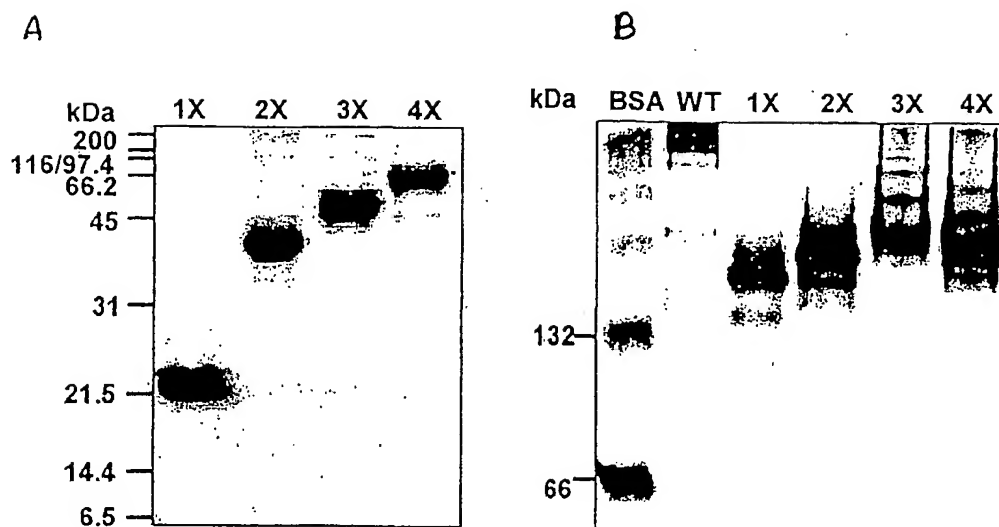
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FIG. 15



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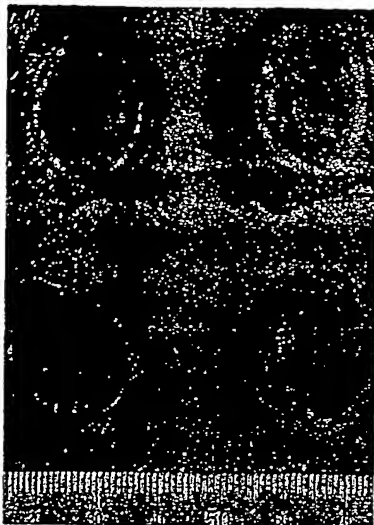
FIG. 16



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FIG. 17

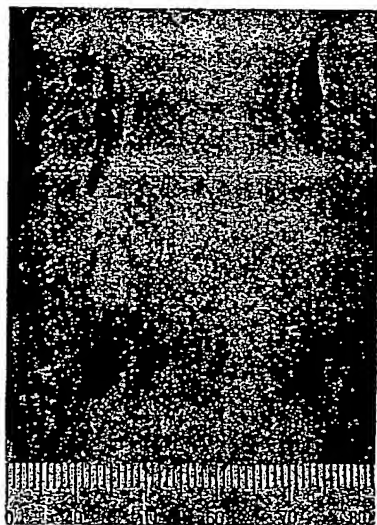
A



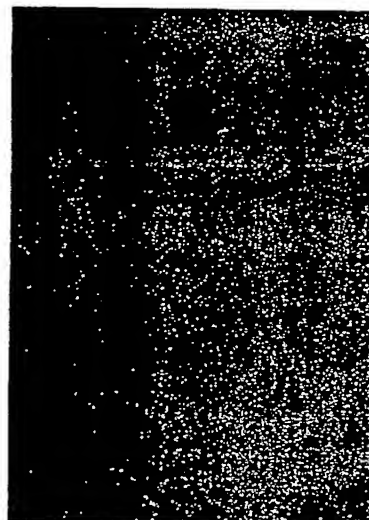
B



C

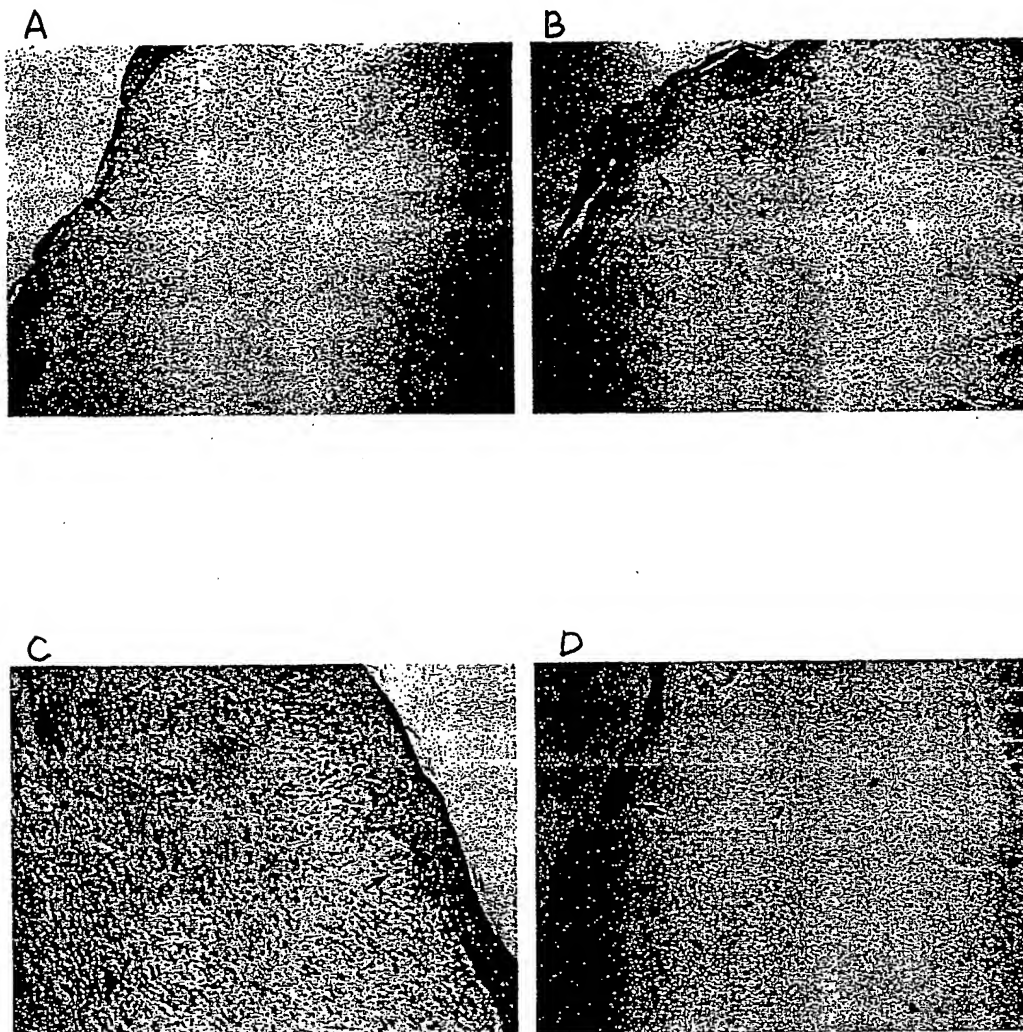


D



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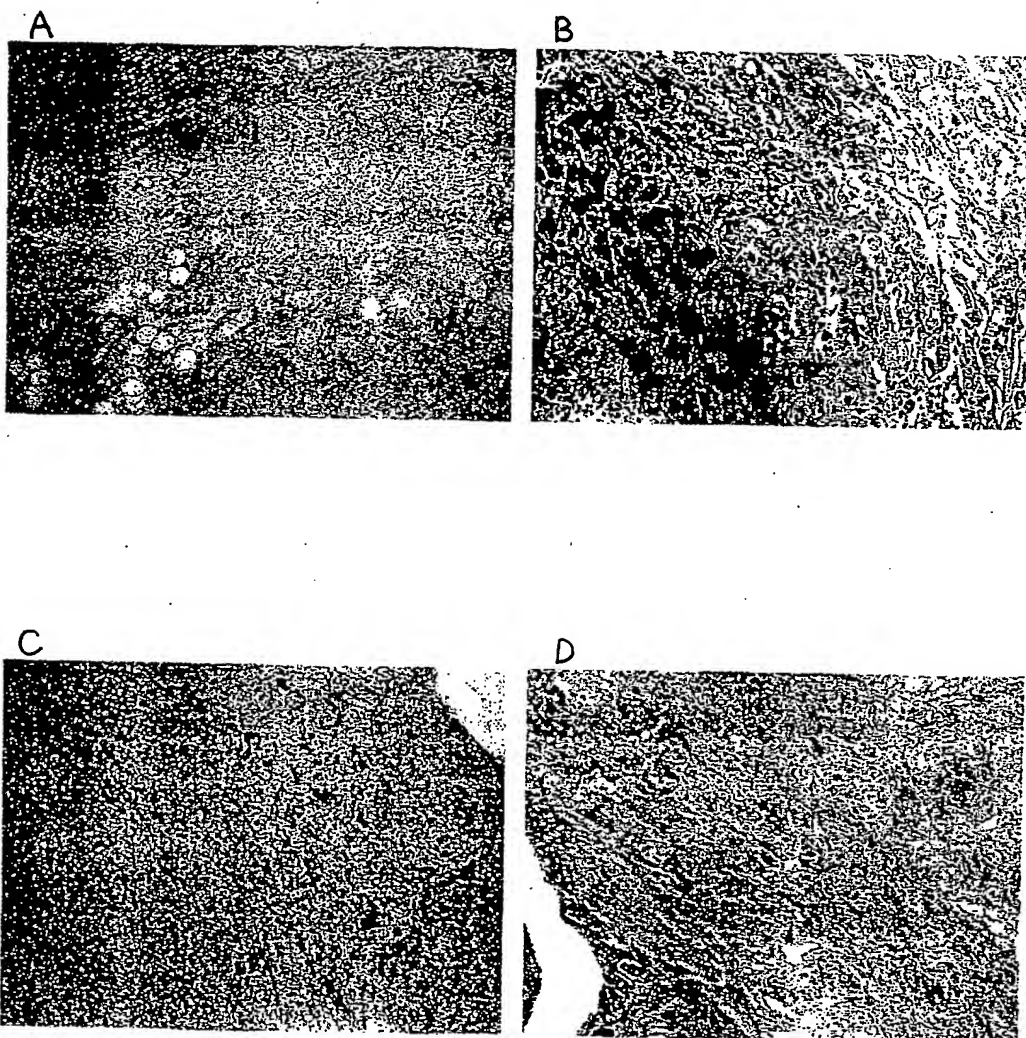
FIG. 18



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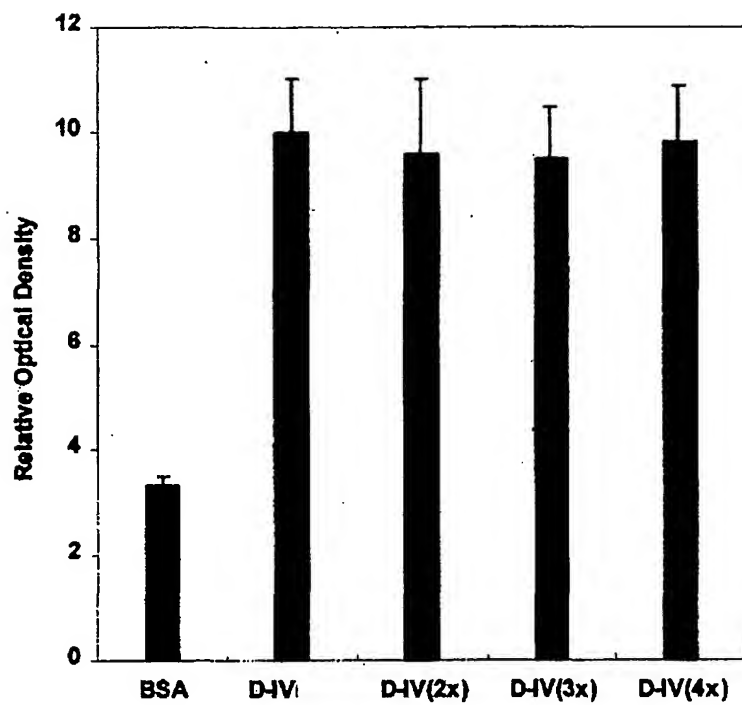
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FIG. 19



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FIG. 20



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FIG. 21

